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Age-dependent Changes Related to Reproductive Development in the Odor Preference of Blowflies, *Phormia regina*, and Fleshflies, *Boetteherisca peregrina*

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ABSTRACT—We investigated the preference for the odor of decaying meat in a T-maze using female and male blowflies, *Phormia regina*, with one of two nutritional histories (protein-containing or protein-free diet), and compared them with fleshflies, *Boetteherisca peregrina*. The preference showed age-dependent changes, which differed according to sex and nutritional history. Females in both nutrition groups showed a high preference for the odor. The preference of protein-fed females showed cyclic variations corresponding to the ovarian cycle, while the protein-deficient group, which never oviposited having immature eggs, did not exhibit cyclic changes and their preference remained high. These results suggest that the preference for the odor may be associated with the ovarian cycle. In males, both nutrition groups showed significant changes with age. The preference of protein-deficient males changed similarly to that of females, whereas protein-fed males were not attracted by the odor. This also suggests that the preference for the odor may be related to reproductive development. With *B. peregrina*, which has different reproductive natures from *P. regina*, the age-dependent changes in the preference did not differ so markedly with sex and nutritional history as with *P. regina*.

INTRODUCTION

The blowfly, *Phormia regina* (Meigen), needs decaying meat as an essential protein source for the development of eggs and reproductive glands in the early days of adulthood, and as an adequate substrate for subsequent oviposition. It can survive on carbohydrates and water only, and completes its normal life span [18]. However, without dietary protein, its eggs and accessory reproductive glands stop developing at three days after eclosion [15, 21], and juvenile hormones [24] or ecdysteroids [23] are not produced. Mated females lay their eggs on decaying meat, whereas they seldom lay on fresh meat. The protein consumption of female blowflies is known to reach a peak during egg development and after oviposition [4]. In males, a moderate amount of protein is consumed during the first day or two after eclosion [4]. Protein uptake is likely to be closely connected to the reproductive development or cycle in both female and male blowflies.

The mechanism of carbohydrate intake has been well studied from both behavioral and physiological aspects [12]. With protein uptake, however, much less is known about what sensory inputs are necessary and what mechanisms regulate it [4, 16, 17]. Olfaction is likely to play an important role in insect behaviors such as feeding and mating. Blowflies of the genus *Calliphora* have been shown to have carrion receptors which specifically respond to the odor of decaying meat [6, 14]. It is supposed that the ability to detect the odor of decaying meat is indispensable to blowflies' lives. Thus, the response to the odor of decaying meat may change during the course of protein feeding and with the reproductive cycle.

Various studies have indicated that chemosensory functions of insects interact with the neuro-hormonal changes associated with the reproductive cycles [5]. For example, in blowflies, the sensitivity of the taste labellar chemosensilla varies depending on the ovarian cycle or related factors [1, 2, 19, 20]. In olfaction, the antennal receptors of mosquitoes have been shown to be associated with the reproductive cycle [9~11]. However, in blowflies, though age-dependent change in olfactory sensitivity has been observed [7, 8], the interaction with the reproductive cycle is not clear.

P. regina is a convenient species to use in studies of the putative factors influencing chemosensory functions, since it is easy to produce two different reproductive states in flies with the same sex, by feeding them protein-containing or protein-free diet. In this study, we investigated the preference for the odor of decaying meat using female and male blowflies, *Phormia regina*, of different ages, with one of two nutritional histories (protein-containing or protein-free diet). In addition, we compared the preference of *P. regina* with that of fleshflies, *Boetteherisca peregrina*, the reproductive nature of which differs from that of *P. regina*. We found that the preference for the odor changed with age and depended on sex and nutrition, suggesting that it may be related to the reproductive functions in both females and males.

MATERIALS AND METHODS

Animals

Blowflies (*Phormia regina*, Meigen) and fleshflies (*Boetteherisca*

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peregrina) were reared in this laboratory. Larvae of *P. regina* were raised on artificial medium (5 g of casein, 5 g of dry yeast, 0.1~0.2 g of agar powder and one drop of lanolin in 36 ml of a solution, one liter of which contained 0.45 g of NaCl, 0.01 g of KCl, 0.013 g of $CaCl_2 \cdot 2H_2O$, 0.01 g of $NaHCO_3$, 0.382 g of $NaH_2PO_4 \cdot 2H_2O$ and 0.3875 g of K_2HPO_4). Larvae of B. peregrina were given pork liver. Larvae and pupae were kept in the dark. Adult flies, separated according to sex within 24 hr after eclosion, were kept in a light:dark cycle of L12:D12. The temperature was maintained at 23±1°C. Adults were raised on 2 kinds of diet. The protein-fed group of *P. regina* was raised on a diet of water, brown sugar and pork liver. That of *B. peregrina* was given minced pork meat instead of pork liver. The protein-free groups of both species were fed on water and 0.1 M sucrose solution. The age of flies is designated as day 0, 1, 2, etc, day 0 being the day of eclosion. The protein-fed group of female *P. regina* was divided further into two groups, mated and unmated. To obtain mated flies, the same number of females and males were put into a cage on day 4 and reared together thereafter. *B. peregrina* females were used without mating.

Determining odor preference in a T-maze

Preferences for the odor were tested in a T-maze, which consisted of a central chamber (40×40×35 mm) and two choice tubes (33×400 mm) (Fig. 1). The apparatus was made of transparent acrylic plates and tubes. One of the choice tubes was attached to the odor source and the other was left open. The right/left orientation of odor and control was randomized for each test. As the odor source, we used about 30 g of decaying meat (minced beef stored at 23°C for 1 week).

Flies were starved by giving only water for 24 hr before testing. Twenty to 40 flies for one trial were shaken into the central chamber, which was shut off from the choice tubes by thin plates. A vacuum hose was attached to the central chamber and air was drawn through by a small pump (6 liters/min). Then, the plates were slid up and the odor of decaying meat or fresh air was allowed to flow through the choice tubes. After 2 min, the plates were slid down and the flies which had moved into the choice tubes or remained in the central chamber were counted. Each fly was used once only. The ages of the flies used were 1 to 12 days for *P. regina* and 1 to 9 days for *B. peregrina*. The numbers of female and male blowflies with one of two nutritional histories were 72 to 328 and tests were done four to ten times for each age. For fleshflies, 43 to 267 were used at two to eight tests. Each series of experiments for each group was done at random. Tests were performed at the same time of the day (10:00 a.m. to 3:00 p.m.) from May to October. Ambient temperatures were 24±2°C and relative humidities were >70%.

Acetic acid or alcohol are attractants for flies at lower concentrations but deterrents at higher concentrations [12]. When we used a high concentration of acetic acid as the odor source (100 μl of 10 M solution on 5×5 cm filter paper), flies moved into the choice tube opposite to the odor source. At low concentration (10^{-2} M), flies tended to go to the odor source. Thus, the preference for the odor was indicated by the number of flies moving into the choice tube on the odor side.

When we used decaying meat as the odor source, 20% to 50% of the flies remained in the central chamber. In 2 min experiments, once flies had gone into one of the choice tubes, they were seldom observed to return to the central chamber or to go into the other choice tube. We estimated the preference for the odor by the ratio of the total numbers of flies moving into the odoriferous tube in all trials to the sum of those in both choice tubes, excluding the flies remaining in the central chamber. These ratios were calculated for each age, sex and nutrition group.

RESULTS

We investigated the preferences for the odor of decaying meat in female and male blowflies, *Phormia regina*, with one of two nutritional histories at each day after eclosion. The preference in each group of flies changed in an age-dependent manner, which also depended on nutritional history (protein-containing or protein-free diet) and sex. We give below full details of the age-dependent changes of *P. regina* and a comparison with those of fleshflies, *Boetteherisca peregrina*.

Odor preferences of blowflies

Females:

On the day after eclosion (day 1), females were not attracted to the odor of decaying meat, but subsequently their preference increased (Fig. 2A). The age-dependent change in preference in the protein-fed group showed cyclic variations between 60 and 90% (Fig. 2A). The changes in preference with age were significant between days 1 and 2, days 3 and 4 ($P<0.01$: Chi-squared test), days 4 and 5, and days 9 and 10 ($P<0.05$). In these experiments, the protein-fed flies were mated on day 4 and were observed to oviposit mainly on days 5~6, 8 and 10. A few eggs were also laid on day 11. The cycle of change in the preference corresponded to the ovarian cycle. The preference reached a peak before the first oviposition. During the ovipositions, the preference

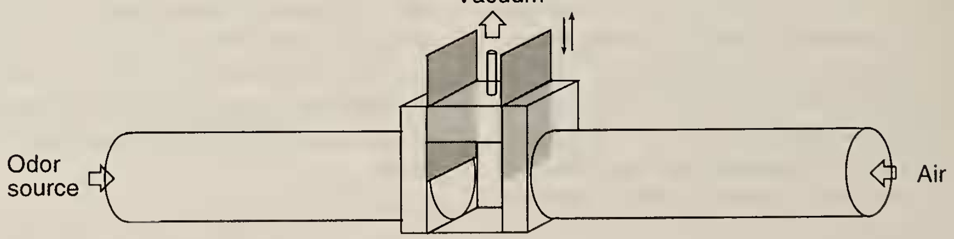

Fig. 1. T-maze. The maze consists of the central chamber and two choice tubes. The central chamber is shut off from the choice tubes by thin plates. One of the choice tubes is attached to the odor source. Suction applied at the central chamber draws air through both choice tubes.

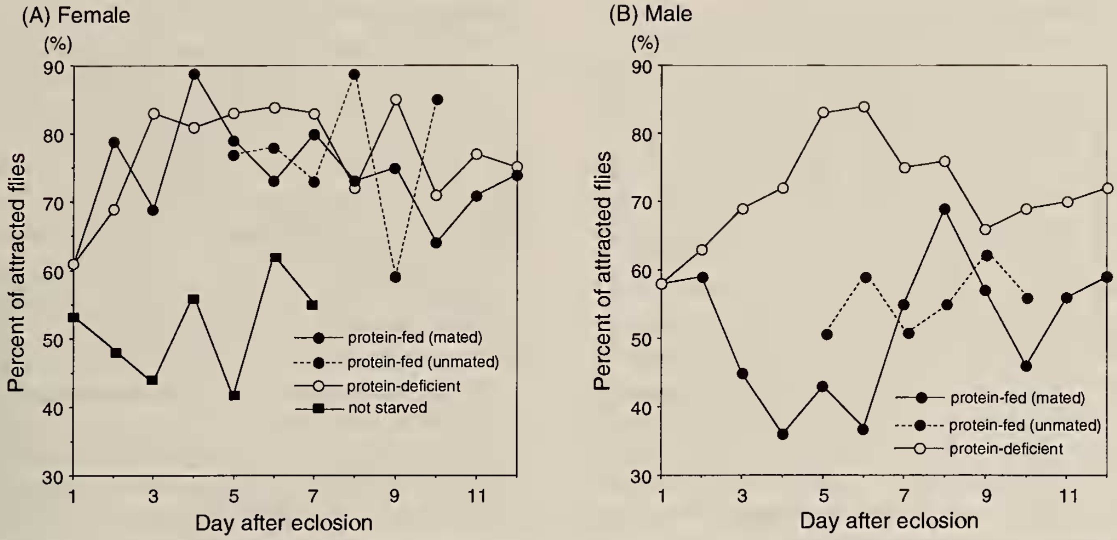

FIG. 2. The preference for the odor of decaying meat of female (A) and male (B) blowflies, *P. regina*. The odor preference of female and male blowflies is shown for each day and each nutrition group (protein-fed and protein-deficient). Results are expressed as the ratio of flies moving into the odoriferous tube to the sum of those in both choice tubes, excluding flies remaining in the central chamber.

decreased and subsequently increased. When flies were not mated, we also observed oviposition on days 6~7, 9 and 12, but the ovarian cycle was one day later than that of the mated flies. In unmated females, the age-dependent change in preference also showed cyclic variations (Fig. 2A). However, significant differences were found between mated and unmated females at days 8, 9 ($P<0.05$) and 10 ($P<0.01$). The cyclic variations of unmated females were one day later than those of mated flies, as was the ovarian cycle. In addition, the range of variation was larger than that of the mated females ($P<0.05$ between days 7 and 8, and $P<0.01$ between days 8 and 9 and days 9 and 10).

In the protein-deficient females, the preference increased with age and reached a peak on day 3; it then remained high but showed a slight tendency to decrease (Fig. 2A). As a whole, the preference of protein-deficient females was a little stronger than that of protein-fed flies (Fig. 2A), showing significant differences at days 3, 6 and 9 ($P<0.05$). Protein-deficient blowflies were never observed to mate or, of course, to oviposit. In contrast with the age-dependent change in protein-fed females which showed cyclic variations corresponding to the ovarian cycle, the preference in the protein-deficient group remained high from day 3 to day 7 and then showed similar cyclic variations between days 8 and 12 ($P<0.05$ between days 2 and 3 and days 7 and 8). There was no correlation between the changes of preference with age of both nutrition groups (the Pearson correlation coefficient was 0.55).

In the above experiments, the flies had been starved for 24 hr before testing. We also measured the preference for the odor daily using the same group of females without prior starvation. Their preference was about 50% and lower than that of the starved flies ($P<0.01$ on days 2~5 and 7) (Fig. 2A). However, their preference increased to 60% during oviposition ($P<0.01$ bewteen days 5 and 6), while the preference of starved flies decreased during oviposition and subsequently increased.

With protein-fed females, the activity in a T-maze (the proportion of flies moving into both choice tubes to all flies used) was 50% at the time of eclosion and increased to about 80%, then decreased thereafter to about 60%. Protein-deficient females tended to show slightly higher activity than the protein-fed flies, but the difference was significant only on day 8 ($P<0.05$).

Males:

With male blowflies, the age-dependent change in the odor preference showed a significant difference between the 2 nutrition groups (on days 3~6, $P<0.01$ and on day 7, $P<0.05$) (Fig. 2B). On day 1, males were not attracted to the odor as much as females. Thereafter, the preference of protein-deficient males increased gradually and reached a peak on day 6 followed by a slow decrease (Fig. 2B). These changes in preference with age were not significant throughout the tested period ($P>0.05$).

On the other hand, the preference of protein-fed males decreased from day 2 ($P<0.05$ between days 2 and 3) (Fig. 2B). Protein-fed males were not greatly attracted to the odor of decaying meat, except on days 2 and 8. They were even repelled by the odor on days 4 and 6. When flies were not mated, the preference was stronger than that of mated males, but there was no significant difference between mated and unmated males except at day 6 ($P<0.05$) (Fig. 2B).

The degree of activity was similar in both nutrition groups, increasing to about 70% gradually from day 0 (43%) and decreasing later to about 50%.

We then compared the preferences of the two sexes with the same nutritional history. Protein-fed females and males

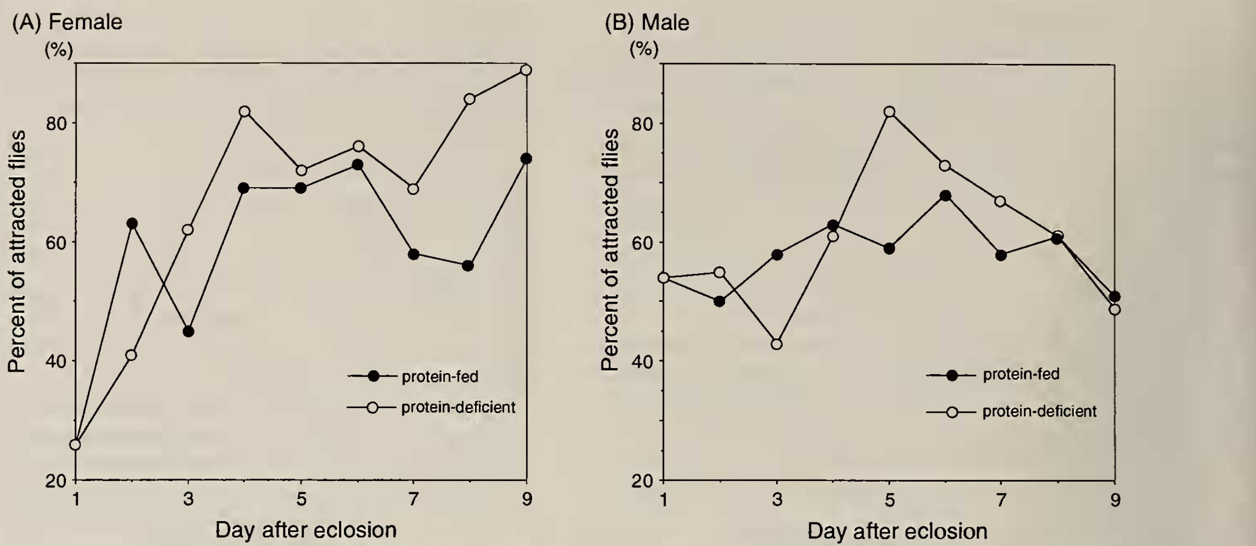

Fig. 3. The preference for the odor of the decaying meat of female (A) and male (B) fleshflies, *B. peregrina*. The preferences are shown for days 1 to 9 in each nutrition group. Results are expressed as described in Fig. 2.

differed significantly. The preference of females was much stronger than that of males (on days 2~7, $P<0.01$; on days 9 ~12, $P<0.05$) (Fig. 2). On the other hand, protein-deficient flies showed a difference between the sexes only on day 3 ($P<0.05$) and day 9 ($P<0.01$), though the change in females was more rapid and their preference remained high longer (Fig. 2). Correlation was found between the changes of preference with age of female and male protein-deficient flies (the Pearson correlation coefficient was 0.64). Females were slightly more active than males and, the difference became greater with time.

Odor preference of fleshflies

We made the same tests using fleshflies, *B. peregrina*. In this species, the preferences of each group also showed age-dependent changes (Fig. 3).

Females:

Both nutrition groups of female fleshflies showed similar age-dependent changes in their preference (Fig. 3A). The preferences increased with age and tended to remain high during the period we examined. There was no significant difference between both nutrition groups except on day 4 ($P<0.05$) and day 8 ($P<0.01$), though the preference in the protein-deficient group was a little greater than that of the protein-fed flies. The age-dependent changes in female fleshflies were similar to those in blowfly females, but fleshflies did not show such clear cyclic variations as the blowflies did.

Males:

The age-dependent change in protein-fed male fleshflies was not striking (Fig. 3B). Their preference remained low, but they were not repelled by the odor, in contrast to the equivalent group of *P. regina*. In addition, no significant difference was found between the nutrition groups of *B. peregrina* males, except on day 5 ($P<0.01$) (Fig. 3B). Nutrition had little effect on the age-dependent change of preference of males as well as of females. With the same nutritional history, the difference between the sexes was small (Fig. 3). In the protein-deficient group, the differences were found on days 4, 8 and 9 ($P<0.01$) (Fig. 3). No difference was observed between males and females in the protein-fed group, except on day 1 ($P<0.05$), in marked contrast to *P. regina*. The degree of activity of all 4 groups of fleshflies was similar to that of each other and to that of the equivalent group of blowflies.

DISCUSSION

Our results showed that the odor preference of *P. regina* exhibited age-dependent change influenced by sex and nutrition.

Both nutrition groups of females showed a strong preference. The reason that females are attracted by the odor of decaying meat is thought to be for feeding and oviposition. Two reasons are likely to induce blowflies to feed: First, they must ingest protein as adults to develop their eggs and accessory reproductive glands completely [15, 21], and they need decaying meat as protein food sources. Next, they search for decaying meat in order to satisfy their hunger because they have been starved for 24 hr before the experiments.

Belzer indicated that the peaks of female protein consumption corresponded to the ovarian cycles [4]. Thus, it is reasonable that the preference for the odor, which is probably closely connected to protein feeding, should change with the ovarian cycle. We found that the preference of protein-fed females did indeed show cyclic variations corresponding to the ovarian cycle. In addition, unmated protein-fed females showed cyclic variations one day later than those of the mated flies, while their ovarian cycles also occurred one day later than those of mated individuals. These facts suggest that the preference for the odor may reflect the need for protein feeding corresponding to the ovarian cycle. This is also supported by the fact that the age-dependent change of protein-deficient females, which never oviposit, did not show correlation with that of protein-fed flies. Furthermore, the fact that the preference of the protein-deficient females reached its peak on day 3 and remained high thereafter suggests that they seek for a protein source in order to complete the maturation of their eggs. This is also suggested by the fact that the preference of protein-deficient females was somewhat stronger than that of the protein-fed flies. In addition, we observed that protein-fed flies began to mate on day 4, whereas protein-deficient flies were never observed to mate. The maturation of eggs or glands is likely to influence the mating behavior as well as the odor preference of the flies.

Blowflies are also thought to be attracted by the odor of decaying meat as a means of satisfying their hunger, regardless of the ovarian cycle. The period of starvation before testing is likely to have increased their preference for the odor, since the preference of unstarved females was weaker than that of starved females.

Moreover, blowflies must search for appropriate oviposition sites. Olfaction has been shown to be very important in the oviposition behavior of blowflies [3]. Our previous experiments showed that flies were able to choose the oviposition site by detecting the odor of decaying meat (paper in preparation). Blowflies are thought to recognize the odor of decaying meat as indicating a suitable oviposition site. We observed that the preference reached a peak after oviposition and decreased during oviposition. It is likely that the preference for the odor primarily reflects the need for protein corresponding to the ovarian cycle, not site-seeking for oviposition. However, in unstarved females, the preference increased during oviposition, supporting the association with site-seeking for oviposition. Stoffolano et al. suggested that during the first ovarian cycle females obtained protein for oögenesis from another source than the oviposition substrate [22]. Thus it is possible that the protein feeding associated with the ovarian cycle and that relating to site-seeking for oviposition may be manifested differently in the odor preference detected in a T-maze.

On the other hand, the preferences were strong in both nutrition groups and the age-dependent change observed with protein-deficient females was identical to that of protein-fed flies after day 7. Therefore, the odor preference may also correlate with some age-related factors, which are probably independent of the ovarian cycle. Previous studies have demonstrated that olfactory sensitivity in female blowflies increases as the females age, regardless of their feeding history [7, 8]. This age-dependent increase is likely to correlate with other features of maturation in the early days after eclosion, such as development of the flight muscles [8]. In addition, the age-related decrease in sensitivity was suggested to be due to an increasing number of inoperative sensilla [19, 20]. It is not clear whether the odor preference in females is related to these phenomena.

In males, the age-dependent change in preference differed significantly depending on nutrition. Protein-deficient males showed increasing preference with age, as did the females, whereas, interestingly, protein-fed males were not attracted by the odor but rather repelled by it. Males also need dietary protein in adulthood for the development of their reproductive glands [21]. In addition, males were shown to consume a moderate amount of protein during the first day or two after eclosion but little or none thereafter [4]. The preferences of both nutrition groups on days 1 and 2 are considered to correspond to protein consumption in the early period. The preference of protein-deficient males, which subsequently increases, might result from the flies searching for protein food sources to develop their glands. Thus, the odor preference of males is probably related to their reproductive development. However, we do not fully understand why protein-fed males were not attracted by the odor and even repelled by it, although they had been starved before the experiments. As a rule, among calliphorids, males do not appear to frequent carrion or live animals to the same extent as females [22]. In protein-fed males with completely developed reproductive glands, some other behavior such as mating is likely to override the need to search for food. If the odor of conspecific females is used as the stimulus, the odor preferences of two nutrition groups of males may be reversed. Stoffolano indicated that sex influenced the sensitivity change in sugar receptors of blowflies but not in that of the salt cells [20], probably because of the metabolic difference related to reproductive functions. The difference in the effects of nutritional history on the preference of both sexes may also be influenced by the metabolic difference by sex.

In fleshflies, *B. peregrina*, the age-dependent changes in odor preference were not so strongly different between the sexes and nutrition groups. *B. peregrina* is ovoviviparous, unlike *P. regina*. Mating was observed to begin on day 2 or 3 after eclosion even with protein-deficient fleshflies, as well as with protein-fed flies, whereas protein-deficient *P. regina* was never observed to mate. Oviposition was not observed until day 10. The fleshflies *Sarcophaga bullata*, which are ovoviviparous like *B. peregrina*, are considered to be incipient autogenous and protein-free diet leads to egg maturation though not completely [15]. A protein-free diet may not have such a critical influence on oögenesis of *B. peregrina* as it does with *P. regina*. Therefore, the effect of nutrition may only slightly influence the odor preference of both groups of *B. peregrina* females. However, we observed a slightly stronger preference in the protein-deficient group than in the protein-fed flies, and an increase in the preference on day 9 (the day before oviposition), also suggesting the influence of the ovarian cycle. With males, a protein-rich diet did not cause them to be repelled by the odor and, similarly, protein deficiency had only a small effect in increasing the preference for the odor. The reproductive nature of *B. peregrina* differs from that of *P. regina*; therefore, the influence of a protein diet may have a smaller differential effect on the age-dependent changes in each group of *B. peregrina* than it does with *P. regina*.

Thus, it is likely that factors related to the ovarian cycle in females and to the development of reproductive glands in males influence the olfactory sensitivity of the flies. Flies may take advantage of these variations associated with their reproductive functions to seek dietary protein, adapting themselves to achieve adequate behavior at that time. We do not fully understand the mechanism regulating olfactory behavior; there may be a central mechanism involving endocrine and neural systems which regulates the olfactory sensitivity. Juvenile hormone, which plays an important role in controlling oögenesis in many insects [13], has been suggested to increase the sensitivity of labellar salt cells in blowflies [1]. A humoral mechanism related to the reproductive cycle is likely to affect the sensitivity of olfactory receptor. Further physiological investigations are necessary to clarify the regulatory mechanisms.

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